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URINARY METHYLMALONIC ACID AS AN INDEX OF

VITAMIN B_{12} METABOLISM

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "METHYLMALONIC ACID EXCRETION AS AN INDEX OF VITAMIN B_{12} DEFICIENCY", submitted by Joan Elizabeth Sutherland Dakin in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Methylmalonic acid, a metabolite of propionic acid, has been reported (1,2,3) to be increased in the urine of patients with untreated pernicious anemia. In order to study methylmalonic acid excretion and vitamin B_{12} deficiency, a quantitative method for the determination of methylmalonic acid in urine was developed. The method uses the technique of thin-layer chromatography on silica gel of an ether extract of urine. The normal urinary excretion of methylmalonic acid by this method was determined to be less than 10 mg. per twenty-four hours.

In twenty-three cases of clinically proven untreated pernicious anemia, urinary levels of methyl-malonic acid were found to be increased. The level of methylmalonic acid returned to normal in response to vitamin B_{12} therapy. These cases also exhibited low Co^{60}-vitamin B_{12} absorption. There appeared to be no correlation between the level of methylmalonic acid and the degree of neurological complication. In some cases, the urinary methylmalonic acid was elevated before hematological abnormalities developed.

As pernicious anemia from a biochemical point of view can be considered to be a defect in nucleoprotein synthesis, it was of interest to determine urinary levels

of 4(5)-amino-5(4)-imidazolecarboxamide nucleotide, an

important intermediate in the synthesis of purine nucleo-

tides. This was done in the patients with pernicious

anemia and levels were found to be only occasionally

elevated. This is at variance with reports (5,6) of

consistent elevations of urinary 4(5)-amino-5(4)-imidazole-

carboxamide in vitamin B_{12} deficiency.

ACKNOWLEDGEMENTS

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I. INTRODUCTION

Pernicious anemia is a disease of vitamin B_{12} deficiency. The classical signs of vitamin B_{12} deficiency include megaloblastosis (due to arrested nucleoprotein synthesis), anemia, neuromyelopathy, encephalopathy, glossitis and alimentary disturbances. The laboratory findings include specific hematologic abnormalities (hypersegmentation of the polymorphonuclear neutrophils, macrocytosis, anemia and megaloblastic erythropoiesis as shown in the bone marrow), histamine-fast achlorhydria, low serum vitamin B_{12} levels and a subnormal absorption of radio-active vitamin B_{12} (Schilling test). The condition responds to injection of vitamin B_{12}, the reticulocyte count being a measure of the hematopoietic response.

In 1962, Cox and White (1) first reported the increased level of methylmalonic acid (MMA), a metabolite of propionic acid, in the urine of patients with pernicious anemia in relapse and suggested that this measurement might serve as an index of vitamin B_{12} deficiency. They found that normal controls and patients with normal serum B_{12} levels had MMA excretions not exceeding 3.8 mg. in twenty-four hours (2). In addition to the markedly increased urinary excretion of MMA in patients with pernicious anemia, it was found that all patients with low serum vitamin B_{12} levels had an increased excretion of MMA, although this was less noticeable in

patients without a disordered erythropoiesis. It was found that, although a constant relationship exists between sub-normal serum B_{12} levels and increased urinary excretion of MMA, there is no proportionate relationship. These authors used a gas chromatographic separation coupled with isotope dilution to estimate urinary MMA.

In 1963, Barness (3) reported a urinary excretion of 500 mg. per day of MMA in one case of pernicious anemia. Following intramuscular doses of vitamin B_{12}, MMA could not be detected in the urine. Barness used two-dimensional paper chromatography to separate MMA from other urinary organic acids. Elution of the acid, followed by titration, gave quantitative results. The excretion level reported of 500 mg. per day in pernicious anemia is five times higher than the highest level reported by Cox and White in five cases of untreated pernicious anemia. This may suggest a lack of specificity in the paper chromatographic procedure.

In view of the interest in the excretion of MMA in vitamin B_{12} deficiency, it was proposed to attempt to develop a simple method for the measurement of MMA in urine. The usefulness of this measurement as an index of vitamin B_{12} deficiency could be partially assessed by comparison with serum vitamin B_{12} levels and results obtained from Schilling tests. Also, Cox and White suggested that there may be a relation between the neurological complications of vitamin B_{12} deficiency and the MMA excretion (reflecting elevated serum MMA). It was thought that the possibility of such a

relationship should also be investigated.

As further evidence of the interest in vitamin B_{12} metabolism, there have been several reports (4,5,6) of increased urinary excretion of 4(5)-amino-5(4)-imidazole-carboxamide (AIC) in patients with vitamin B_{12} and/or folic acid deficiency. AIC in the ribotide form is a key intermediate in the biosynthesis of the purine moiety of nucleic acids. It was, therefore, decided to measure urinary levels of AIC.

II. VITAMIN B_{12}

Vitamin B_{12}, the anti-pernicious anemia factor or extrinsic factor of Castle, was first isolated from liver in 1948. In 1958, Barker et al (7) isolated three vitamin B_{12} containing coenzymes from microbial sources. Vitamin B_{12} is unique among the vitamins in that it is synthesized only by microorganisms.

A. CHEMISTRY

Vitamin B_{12}, $C_{63}H_{88}O_{14}N_{14}PCo$, molecular weight 1356, is a red crystalline compound and has a structure as shown in Figure I. It consists of two major structures, a planar group resembling a porphyrin ring, and a nucleotide nearly perpendicular to the planar group. The planar group consists of four reduced and extensively substituted pyrrole rings (A - D) linked to a central cobalt atom. The pyrrole rings are connected to one another by methene bridges except for a direct linkage between the αcarbons of rings A and D.

VITAMIN B$_{12}$
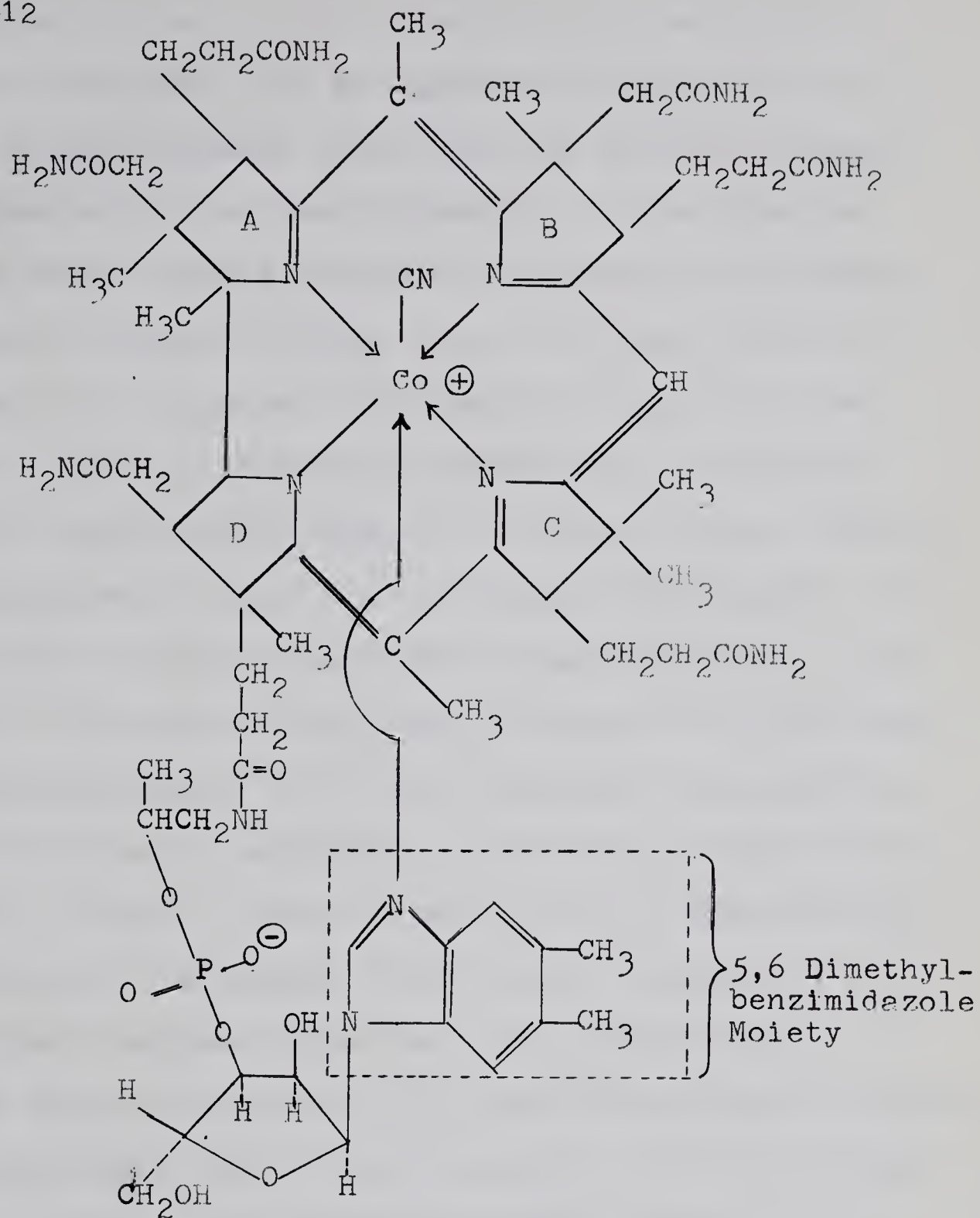
COENZYME
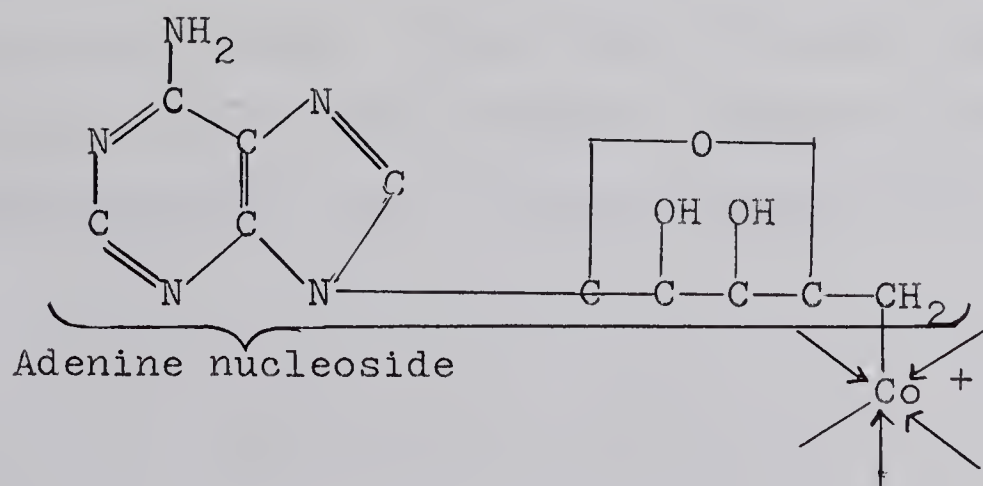
FIGURE I.   Structure of Vitamin B$_{12}$ and the B$_{12}$ coenzyme.

Below the planar group is the nucleotide, 5,6,-dimethyl-
benzimidazole ribotide. It is connected at one end in a
coordinate linkage between cobalt and one of the nitrogens
of the imidazole ring of benzimidazole. At the other end
there is an ester linkage between the nucleotide phosphate
and a 1-amino-2-propanol moiety that is in turn joined in
amide linkage to a propionic side chain on ring D of the
tetrapyrrole nucleus. Above the planar group, a cyanide
group is also coordinately bound to the cobalt atom. Removal
of the cyanide group results in a compound "cobalamin". The
presence of the cyanide group forms "cyanocobalamin". Sub-
stitution of the cyanide group with a hydroxy or nitro group
forms "hydroxycobalamin" and "nitrocobalamin" respectively.

The cobamide coenzymes are the biologically active
forms of the vitamin. The coenzymes have a 5'-deoxyadenosyl
group in place of the cyanide ligand above the plane. The
three cobamide coenzymes which have been identified all
possess the adenine nucleoside but they differ from one another
in the benzimidazole part of the molecule. In this position
there may be the 5,6,-dimethylbenzimidazole group as in
vitamin B_{12}, an unsubstituted (methyl free) benzimidazole
group or an adenyl group. These three cobamide coenzymes are
designated 5,6,-dimethylbenzimidazole cobamide (DBC),
benzimidazole cobamide (BC) or adenyl cobamide (AC) coenzymes.

B. PHYSIOLOGY

In man, the whole body pool size of vitamin B_{12} is approximately 5 mg. and the metabolic turnover rate about 2.5 µg. per day (8). Clinical symptoms of vitamin B_{12} deficiency do not appear until the body pool is depleted to about 10% of the normal value.

Vitamin B_{12} is absorbed from the ileum and the absorption is dependent upon the presence of intrinsic factor (IF). IF is secreted by the fundus of the normal stomach and while it has not yet been isolated in pure form is thought to be a mucopolypeptide or mucopolysaccharide. The mechanisms of the vitamin B_{12}-IF binding and the absorption of vitamin B_{12} are not known. It has been suggested (9) that there are "receptors" for either IF alone or the vitamin B_{12}-IF complex in the wall of the ileal mucosa, and that there may be two binding sites on the IF molecule, one for vitamin B_{12} and one for the "receptor" in the gut wall. Large molecular complexes, when absorbed, are transported via the lymphatics. Since absorbed vitamin B_{12} travels in the blood stream it is probably absorbed alone rather than in combination with IF. This would suggest a system in the ileum or ileal mucosa that releases vitamin B_{12} from IF. Vitamin B_{12} is transported in the blood stream, 80 - 85% being bound to an alpha globulin, and is stored in the liver.

Megaloblastic anemia is the hematologic result of any of a variety of etiologic factors causing improper

nucleoprotein synthesis. The most common causes are defi-
ciencies of vitamin B_{12}, folate, or both. Vitamin B_{12}
deficiency has been generally classified by Herbert (10) as
resulting from:

1. Inadequate ingestion, e.g. malnutrition,
vegetarians, "vegans". In this case, 5 micrograms of vitamin
B_{12} orally per day is adequate therapy.

2. Inadequate absorption

a. Inadequate secretion of IF

In the absence of IF, practically
none of the 2 - 10 micrograms of vitamin B_{12} present in the
average diet can be absorbed. The vitamin deficiency then
develops over a three to six year period, the time taken to
nearly exhaust the liver stores.

Addisonian pernicious anemia is due
to inadequate IF secretion and is usually associated with
gastric atrophy. Parenteral administration monthly of 250
or 500 micrograms of vitamin B_{12} serves to treat the condition.

b. Functional abnormality of ileum

Vitamin B_{12} deficiency may be due to
inadequate absorption due to a functional or structural
abnormality in the ileum, the main site of vitamin B_{12}
absorption.

c. Inadequate utilization

This cause of vitamin B_{12} deficiency
has not yet been demonstrated but, according to Herbert (10),
will be the inevitable consequence of anti-vitamin B_{12} therapy
of cancer which has been proposed.

C.   ROLE IN METABOLISM

Vitamin B_{12} functions as a cobamide coenzyme in several reactions in animals and microorganisms. In addition, it is involved in DNA synthesis largely due to its interrelation with folic acid.

1.   Enzymatic Reactions Involving Cobamide Coenzymes

a.   Methylmalonyl - CoA mutase (isomerase)

In 1955, Flavin, Ortiz and Ochoa (11) showed that a major pathway of propionic acid utilization in tissues of animals involves the conversion of propionyl-CoA to succinyl-CoA by way of an intermediate compound methylmalonyl-CoA. The series of reactions which occur is shown in Figure II and can be summarized as follows:

$$\text{Propionyl-CoA} + \text{HCO}_3^- + \text{ATP} \xrightleftharpoons{\substack{\text{Propionyl-CoA} \\ \text{carboxylase} \\ \text{Biotin}}} \text{Methylmalonyl-CoA (a)} + \text{ADP} + \text{P}_i$$

$$\text{Methylmalonyl-CoA (a)} \xrightleftharpoons{\substack{\text{Methylmalonyl-CoA} \\ \text{Racemase}}} \text{Methylmalonyl-CoA (b)}$$

$$\text{Methylmalonyl-CoA (b)} \xrightleftharpoons{\substack{\text{Methylmalonyl-CoA mutase} \\ \text{Cobamide coenzyme}}} \text{Succinyl-CoA}$$

$$\text{Succinyl-CoA} + \text{Propionate} \xrightleftharpoons{} \text{Propionyl-CoA} + \text{Succinate}$$

$$\text{Succinate} \xrightleftharpoons{} \text{Oxaloacetate} + 4\text{H}$$

$$\text{Oxaloacetate} \xrightleftharpoons{} \text{Pyruvate} + \text{HCO}_3^-$$

SUM:

$$\text{Propionate} + \text{ATP} \longrightarrow \text{Pyruvate} + 4\text{H} + \text{ADP} + \text{P}_i$$

FIGURE II. Conversion of propionate to succinyl-CoA.

It was shown that dimethyl benzimidazole cobamide (DBC) coenzyme was involved in the isomerization of methyl-malonyl-CoA (b) to succinyl-CoA. This isomerization occurs by an intramolecular shift of the CoA - carboxyl between the α and β carbons of the propionic acid moiety.

It is because of this requirement of the methyl-malonyl-CoA mutase for the DBC coenzyme that in patients with vitamin B_{12} deficiency this reaction is blocked and increased amounts of methylmalonic acid are excreted in the urine.

b. Glutamate mutase (isomerase)

Extracts of Cl. tetanomorphum convert glutamate to mesaconate via β-methylaspartate. The glutamate to β-methylaspartate reaction involves the enzyme glutamate mutase (β-methylaspartate mutase) which requires the adenyl cobamide (AC) coenzyme. This coenzyme was isolated by Barker and was the first of the vitamin B_{12} coenzymes to be isolated. It is the coenzyme of pseudovitamin B_{12} in which adenine replaces dimethylbenzimidazole in the nucleo-tide moiety. The following are the reactions involved.

$$
\begin{array}{ccc}
\begin{array}{l}
COOH \\
| \\
CHNH_2 \\
| \\
CH_2 \\
| \\
CH_2 \\
| \\
COOH
\end{array}
&
\xrightarrow[\text{B_{12} coenzyme}]{\text{Glutamate mutase}}
&
\begin{array}{l}
COOH \\
| \\
CHNH_2 \\
| \\
CH-CH_3 \\
| \\
COOH
\end{array}
\end{array}
$$

$$
\xrightarrow[\text{β-Methylaspartase}]{\pm NH_3}
\quad
\begin{array}{l}
COOH \\
| \\
CH \\
\| \\
C-COOH \\
| \\
CH_3
\end{array}
$$

Glutamate $\qquad$ β-Methylaspartate $\qquad$ Mesaconate
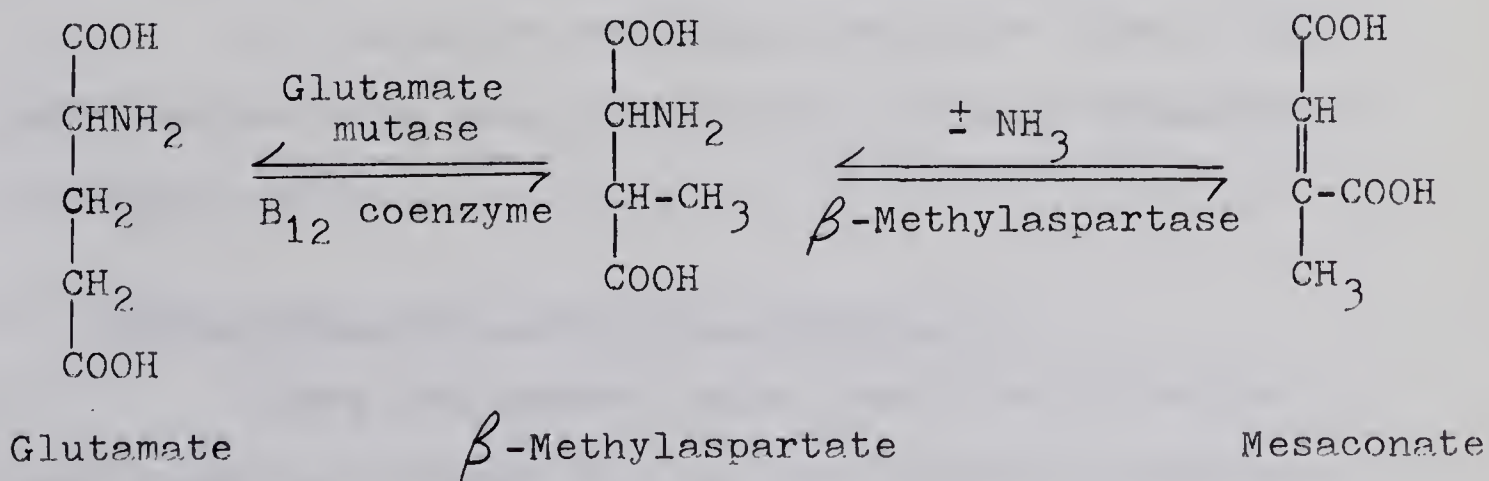
c. Dioldehydrase

Cell free extracts of A. aerogenes catalyze a cobamide dependent dehydration of glycols to the corresponding deoxyaldehyde, specifically 1,2 propanediol to propionaldehyde,

$$CH_3-\underset{\underset{OH}{|}}{CH}-\underset{\underset{OH}{|}}{CH_2} \longrightarrow CH_3-CH_2-CHO + H_2O$$
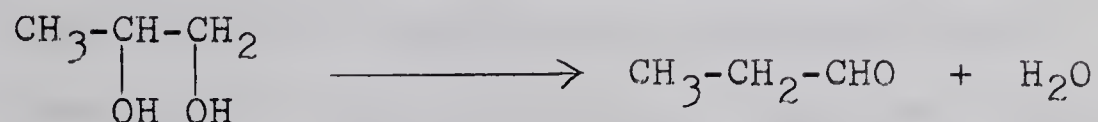
and ethylene glycol to acetaldehyde.

$$\underset{\underset{OH}{|}}{CH_2}-\underset{\underset{OH}{|}}{CH_2} \longrightarrow CH_3-CHO + H_2O$$
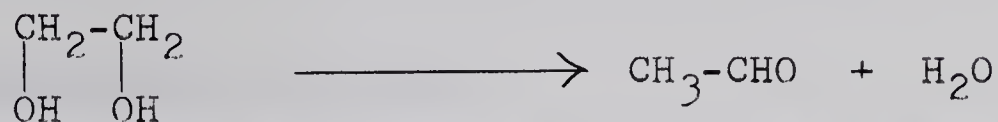
The enzyme, dioldehydrase, of A. aerogenes requires the AC coenzyme as cofactor.

d. Lysine degradation

Cl. Sticklandii and Clostridium ME degrade lysine in the presence of pyruvate to yield one mole each of butyrate and acetate and two moles of ammonia. The reaction involves the participation of a cobamide coenzyme, is inhibited by intrinsic factor, leads to ATP formation and requires a flavin, a disulfide and Fe^{++} ion.

Of the above reactions, only the first, the methylmalonyl-CoA mutase reaction, has been demonstrated in animal cells.

2. Other Reactions Involving Vitamin B_{12}

There are several other reactions in which involvement of vitamin B_{12} has been shown or postulated. Some of these reactions are ones which primarily involve folic acid.

a. Biosynthesis of methionine methyl group

Both vitamin B_{12} and folic acid are implicated in the biosynthesis of the methyl group of methionine.

Homocysteine + N^5-Methyl tetrahydrofolic acid

$\xrightarrow{B_{12} \text{ "enzyme"}}$ Methionine + Tetrahydrofolic Acid (THF)

The enzyme 5-Methyl THF - homocysteine transmethylase catalyzes irreversibly the vitamin B_{12} - dependent transfer of methyl groups with the formation of methionine and THF,

b. Deoxyribose synthesis

It has been suggested (13, 14) as a result of work with cultures of L. leichmannii that vitamin B_{12} may participate in the conversion of ribose to deoxyribose.

c. Thymidylic acid methyl synthesis

O'Brien (15) suggests that vitamin B_{12} is involved in the following reaction.

Deoxyuridylic acid + N^5N^{10} methylene THF $\xrightarrow{B_{12}?}$

Thymidylic acid + Dihydroxyfolic acid (DHF)

d. Purine biosynthesis

Luhby and Cooperman (4) postulate that vitamin B_{12} interacts with folic acid in the formylation of AIC ribotide (AICAR) to formamidoimadazolecarboxamide ribotide (FAICAR).

AICAR + N^{10}-formyl THF $\xrightarrow{B_{12} \text{ "enzyme"}}$ FAICAR + THF

AIC ribotide is an intermediate in the biosynthesis of inosinic acid, the first purine to be synthesized and from which adenine and guanine nucleotides are derived.

D. **BIOCHEMICAL ASPECTS OF VITAMIN B_{12} DEFICIENCY**

A characteristic feature of vitamin B_{12} or folic acid deficiency is the megaloblastic erythropoiesis which results in the production of abnormal erythrocytes. The megaloblast is a morphologic entity characterized by a large cell size, increased ratio of cytoplasmic to nuclear area, a characteristic fine grained chromatin texture and cytoplasmic basophilia. The megaloblast contains a normal or slightly increased amount of DNA and a substantially increased amount of RNA per cell i.e. an increased RNA/DNA ratio. This cell is in a state of unbalanced growth due to impaired deoxyribotide synthesis. DNA replication and cell division are blocked whereas synthesis of cytoplasm (RNA and protein) proceeds normally, hence the RNA/DNA ratio rises. Buchanan (16) suggests that the reactions of the methionine cycle (homocysteine - methionine reaction) are critical in the disordered erythropoiesis of folic acid and vitamin B_{12} deficiencies. In vitamin B_{12} deficiency, the inability to convert N^5-methyl THF to THF results in a trapping of methyl THF (the storage form of folate) with a resultant "functional" deficiency of folate. The folate coenzymes function in the metabolism of "one - carbon" units, principally for the transfer of methyl, methylene, and formyl groups for the synthesis of compounds involved in the biosynthesis of purines and pyrimidines and hence RNA and DNA. In purine biosynthesis the reactions in which folate coenzymes are involved are the formylation

of glycinamide ribotide to formylglycinamide ribotide and
the formylation of AIC ribotide to formaminoimidazole-
carboxamide ribotide. The first reaction depends on
N^5N^{10}-methenyl THF and the second on N^{10}-formyl THF. In
pyrimidine biosynthesis, the methylation of deoxyuridylic
acid to thymidylic acid (the latter the pyrimidine nucleo-
tide which differentiates DNA from RNA) requires N^5N^{10}-
methylene THF.

Luhby and Cooperman (4) on finding an increased
urinary excretion of AIC in vitamin B_{12} deficiency suggest
a block in the reaction

AICAR + N^{10}-formyl THF $\longrightarrow$ FAICAR + THF

in the purine biosynthetic pathway which could account
for the defective nucleic acid synthesis in pernicious
anemia. However, it is only by inference that this may be
a vitamin B_{12} dependent reaction.

The methylmalonyl - CoA isomerase reaction is
not concerned with the abnormal nucleoprotein metabolism
of megaloblastic anemia and it is not known what physio-
logical consequences may result from impairment of this
reaction.

Neurologic symptoms are a feature of 80 to 90%
of all cases of pernicious anemia (17). No known specific
biological action of vitamin B_{12} on the central nervous
system has yet been shown.

The laboratory diagnosis of vitamin B_{12} deficiency
includes the following procedures:

1. Hematological examination of peripheral blood and bone marrow.

2. Determination of vitamin B_{12} concentration in the blood serum.

3. Determination of the presence of free hydro-chloric acid in gastric contents.

4. Measurement of the absorption of radio-active vitamin B_{12} (Schilling test).

There are some problems encountered in some of the above procedures. The serum vitamin B_{12} determination usually involves a microbiological assay. The Schilling test necessitates the use of radio-active material and on occasion may give inconclusive results. It was felt, therefore, that there would be value in the development of a procedure to measure urinary MMA excretion as another index of vitamin B_{12} deficiency.

It was proposed then to investigate the following:

1. The development of a quantitative method to demonstrate the presence of increased quantities of MMA in the urine.

2. The assessment of the excretion of MMA as a diagnostic tool for the diagnosis of vitamin B_{12} deficiency, this to be done by comparison with the Schilling test and the hematological findings.

3. The relation, if any, of the amount of MMA excreted to the neurological symptoms present.

4. The excretion of AIC in vitamin B_{12} deficiency.

III. EXPERIMENTAL

A. DETERMINATION OF URINARY METHYLMALONIC ACID

With the work of Barness and co-workers (3) as
a basis, MMA was first determined by a paper chromatographic
procedure which gave satisfactory qualitative results.
As reported by Barness (3), increased amounts of MMA were
observed in the urine from patients with pernicious anemia.

It was then thought that the advantages offered
by thin-layer chromatography, i.e. better resolution of
components, greater sensitivity, decreased time required
for development, should be applied to the separation of MMA.
As a result systems were developed for qualitative and semi-
quantitative and finally for quantitative estimations of
urinary MMA by thin-layer chromatography.

1. Paper Chromatography

The paper chromatographic procedure followed
was a modification of that of Barness (3). Initially,
a 5 ml. aliquot of a twenty-four hour urine collection
was acidified to pH 1 with 5N H_2SO_4 and continuously
extracted in a 250 ml. ether extractor with 110 ml. of
ethyl ether for three hours. Subsequently, an aliquot
equal to approximately 1% of the twenty-four hour urine
volume (to an even 5 ml. greater than the 1% volume, i.e.
5, 10, 15 or 20 ml.) was acidified and ether extracted for
from four to six hours depending on the volume. The ether
extract was evaporated under a stream of air to approximately

0.5 ml. and this concentrated extract applied to a ten inch square Whatman No. 1 chromatography paper. An ascending two-dimensional system was used with ethanol : ammonium hydroxide : water (80:5:15) as the first solvent and propanol : eucalyptol : formic acid (50:50:20) and water to give persistent turbidity as the second solvent. An overnight run was used for both solvents.

After evaporation of the second solvent the chromatogram was stained with the Altman reagent (18), 10% p-dimethylaminobenzaldehyde in acetic anhydride (1 volume) and acetone (4 volumes). After drying, the chromatogram was heated at 105 - 110°C. for two to three minutes. The yellow spot of methylmalonic acid was easily identified and had an Rf of 0.30 and 0.79 in the solvent systems used.

The solvents used are those suggested by Smith (18) for organic acids and gave good separation. Barness (3) used bromcresol green as the location reagent however it was found in this laboratory that with this reagent the MMA spot was neither well defined nor easily distinguishable from the other organic acids present.

For each sample, a parallel determination was made on an aliquot of the urine to which MMA had been added.

The minimum quantity of MMA detected by the Altman reagent was determined to be approximately 100 ug. under the conditions described. Therefore, by using a minimum of 1% of the volume of urine excreted in twenty-four hours

for extraction, an excretion in excess of approximately
10 mg. or slightly less of MMA per twenty-four hours
could be detected. Results were reported as "none detect-
ed" if no MMA was seen on the chromatogram or "present"
if the yellow MMA spot was observed.

2. Thin-Layer Chromatography

a. Qualitative determination

Thin-layer chromatography of MMA on silica gel
layers was attempted. An aqueous slurry of silica gel
containing 13% $CaSO_4$ as a binder (Merck Silica Gel G)
was spread on 20 x 20 cm. glass plates with an adjustable
spreader* to give a layer 250 microns thick. Silica gel
containing a fluorescent additive as well as the $CaSO_4$
binder (Merck Silica Gel GF_{254}) was also used. The plates
were activated just before use by heating at 110 - 120°C.
for 30 to 60 minutes.

The following solvents were tried:

1. Ethanol : Ammonia : Water (160:10:30) (18)

2. Acetonitrile : Ammonia : Water (100:5:30) (19)

3. Isopropyl ether : Formic Acid : Water
(90:7:30) (20)

4. Propanol : Eucalyptol : Formic Acid (50:50:20)
plus water to persistent turbidity (18)

The following location reagents were also tried:

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* Desaga Apparatus - Brinkman Instruments Incorporated.

1.   Bromcresol Green   (18)

2.   Altman Reagent   (18)

3.   Potassium Ferrocyanide - Ferric Ammonium
     Sulfate Reagent   (18)

4.   Bromcresol Purple   (20)

Of the solvents and location reagents used it
was found that ethanol : ammonia : water as solvent in a
one-dimensional system and bromcresol green as location
reagent gave the most satisfactory results.   In this
system, MMA gave a distinct yellow spot on a blue background,
well separated from other organic acids present in urine.

The initial runs were made using the Desaga tank
lined with solvent saturated filter paper.   The plates
were equilibrated in the tank before allowing the solvent
run to begin.   Rather variable Rf values for MMA (0.20 to
0.30) and a variation in the time required to give a solvent
front of a specific distance were observed.   Subsequently
the CAMAG "Sandwich" Chamber was used which gives a
consistent saturated atmosphere and with which no pre-
equilibration is required.   With this apparatus it was
found that a four hour run gave a solvent front of
approximately 15 cm. and that the Rf of MMA averaged 0.42.
10 µg. of MMA was the minimum amount that could easily be
detected by this procedure using bromcresol green as
location reagent.

Urine samples were ether extracted as for paper
chromatography.   It was found that 2 mg. of MMA was

completely extracted from 10 ml. of solution (correspond-
ing to 200 mg. per liter) in four hours. 2 mg. of MMA
was about 85% removed from 20 ml. of solution after four
hours extraction but was completely removed after six
hours. The length of the extraction time depended,
therefore, on the volume of urine being extracted. The
extract was evaporated to 0.5 ml. and amounts of this
extract varying from 10 to 50 μl. were applied to the
thin-layer plate.

By application of varying amounts of a standard
MMA solution and of varying quantities of the extract of
an unknown urine and visual comparison of the size and
the intensity of the unknown spots with those of the
standards, a semiquantitative estimate of the MMA
present could be made. Four standard spots equivalent
to 20, 40, 60 and 80 μg. of MMA and 10, 20, 25 and 50 μl.
of the unknown extract were routinely applied

Figure III represents a thin-layer chromatogram
on which four standard spots and four amounts of a urine
extract were developed.

b. Quantitative determination

First attempts at quantitation were made on
extracts of the MMA-containing gel scraped off the plate.
The spots were located by one of two methods. A standard
MMA solution was run at the position nearest one side edge
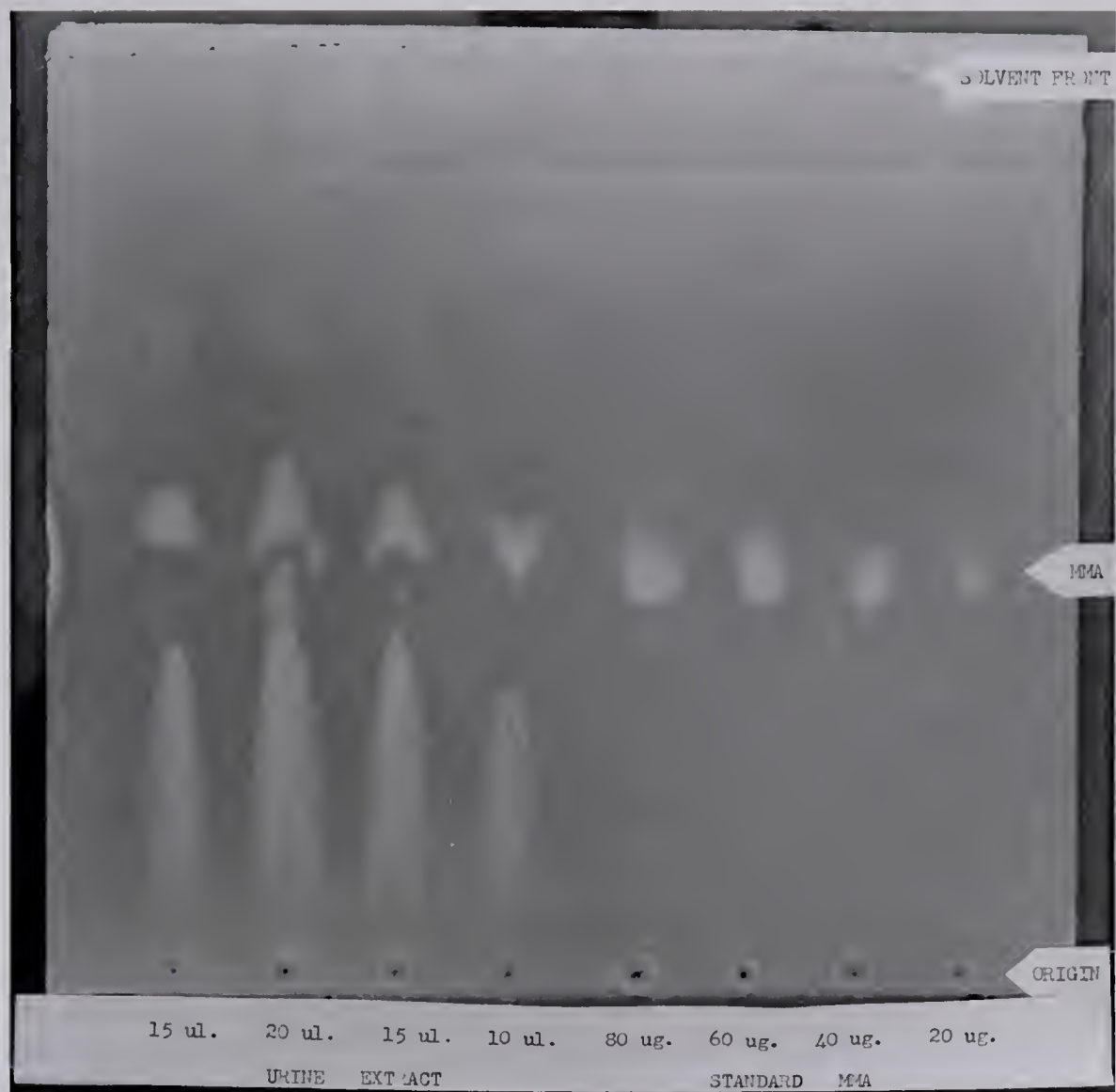
FIGURE III. Thin-layer chromatogram of standard MMA and urine extract.

of the plate. After development, the entire plate but
that area above the point where this standard had been
applied was covered by a second glass plate (uncoated)
and the remaining strip at the edge sprayed with brom-
cresol green and the MMA spot located. Areas of uniform
size corresponding in position to the MMA spot were
removed from the plate. Alternatively, when using the
silica gel containing the fluorescent material, MMA spots
could be located under short-wave ultra-violet light as
spots absorbing the ultra-violet light.

To the MMA-containing gel was added water to
elute the MMA. When desired the silica gel was removed
by centrifugation.

Various approaches to quantitate the MMA by
chemical means were attempted. These included:

1. Titration of the acid

2. Determination of acidity by iodometric
   titration (an acid catalyzed reaction) (21)

3. Determination of the organic acid with a
   copper-benzidine reagent (22)

4. Conversion of the acid to a hydroxamate and
   formation of the colored iron chelate (23)

All showed some promise when applied to aqueous standard
solutions of MMA but none was satisfactory when applied to
the MMA-containing gel or to the eluate. It was also
found that the sensitivity which could be obtained with
aqueous standards was not as great as would be necessary for

the amounts present in urine extracts, i.e. a minimum
sensitivity of 10 - 20 μg. MMA.

Quantitation of MMA was then attempted by means
of densitometry. The problem here was the removal of the
gel in an intact and more or less rigid form in order to
scan it. After being sprayed with bromcresol green, the
gel was sprayed with a plasticizing emulsion (Neatan,
Merck) and allowed to dry. Then broad (eight inch)
cellophane tape was applied directly to the gel and with
care the gel could be removed from the glass plate, and
the MMA containing strip scanned. The instrument used
was the Chromoscan Reflectance Densitometer. Filters
5021 and 5022 (585 and 595 mμ.) and a 0.5 by 20 mm. slit
were used. The tracing obtained had a high baseline with
depressed peaks representing the yellow spots of MMA.
Plotting the area under the curve against the amount of
MMA present gave an approximately straight line in the
20 to 80 μg. region.

Application of the plasticizing emulsion had the
disadvantage of causing a certain lack of uniformity of
the background color and had an overall lightening effect
on the gel.

At this time a commercially prepared 100 micron
thick layer of silica gel containing an inert plastic
binder applied to a flexible plastic support became
available (Eastman Chromagram). The gel adheres quite
firmly to the support and the sheet can be cut as desired

without damage to the gel layer. The Chromagram seemed
most adaptable for quantitation of MMA by densitometric
scanning as it eliminated the need for plastic coating the
gel.

Standards and samples were run on the 20 x 20 cm.
Chromogram in a manner identical to that used with the
glass plates. The time for development was considerably
longer, being about eight hours to give a 15 cm. solvent
front as compared with about four hours for the 250 micron
thick silica gel on glass plates. The Rf of MMA on the
Chromagrams was about 0.45.

It was found that heating the Chromagram at
110 - 120°C. for two to four minutes after spraying with
bromcresol green resulted in a clearer definition of the
yellow MMA spots. A strip containing these spots was
cut out and scanned. Results using the Chromagram were
rather more reproducible than those using the plastic-
sprayed plates. Figure IV shows a graph typical of those
obtained by plotting the area under the curve against the
amount of MMA present.

3. Discussion

While the Chromagrams were much more suitable
for the densitometric scanning, the sensitivity was some-
what less. 20 μg. of MMA could be detected on the Chromagram
with about the same ease as could 10 μg. of MMA on the 250
micron thick layers of silica gel on glass plates. It was
also found latterly that there was some variation from lot
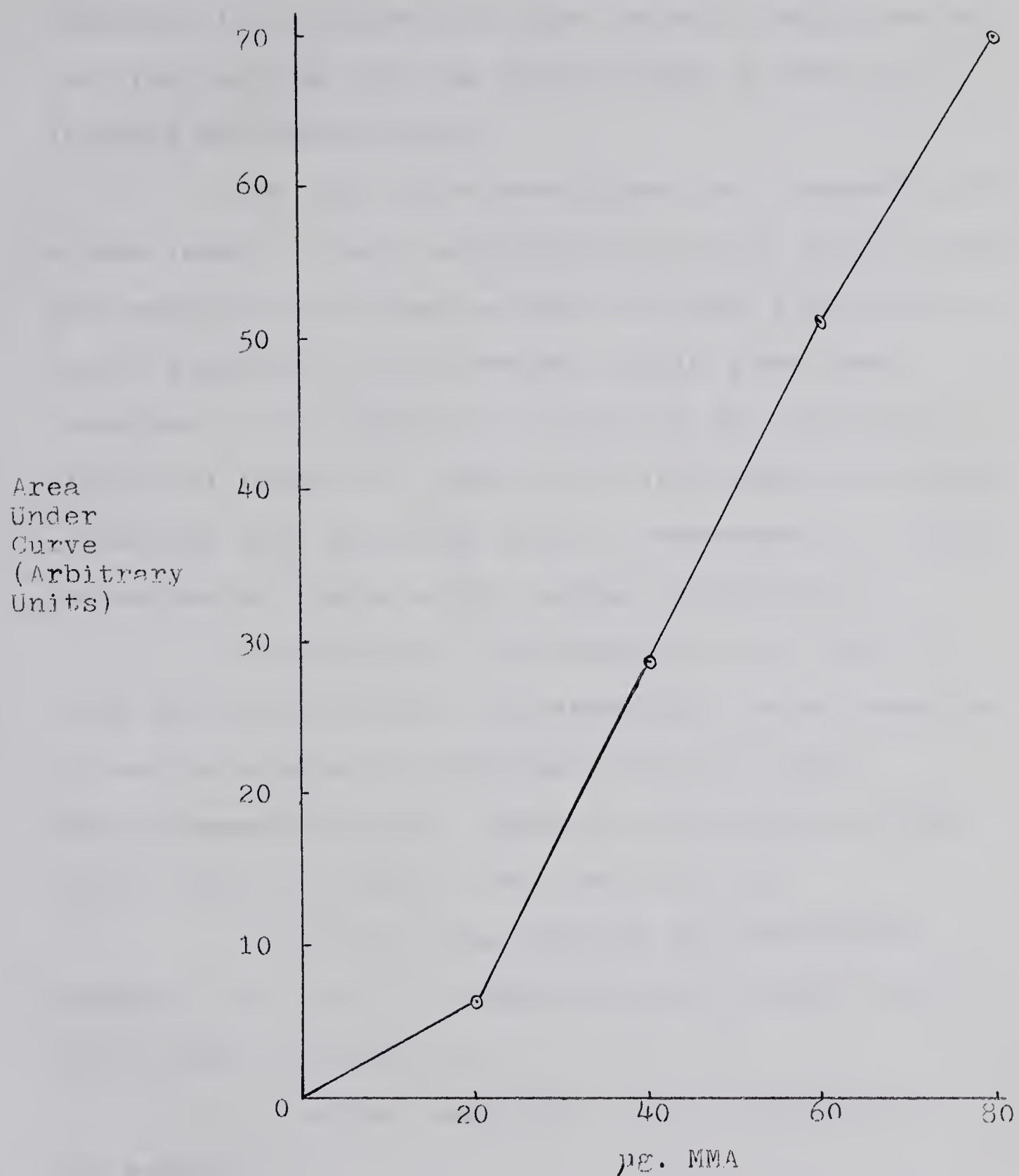
FIGURE IV. Standard curve typical of those obtained by
plotting areas under the curves versus micrograms
of MMA present.

to lot in the time of development required for the
Chromagrams. With most chromatograms, the solvent front
travelled 14 to 15 cms. in eight hours but with one lot
the time required for the solvent front to move this
distance was twelve hours.

With both 250 micron layers and Chromagram 100
micron layers, visual comparison of unknown with standard
gave results very close to those obtained from densito-
metric scanning, on an average, within 5 per cent.
Therefore, it is probable that use of the 250 micron
silica gel layer on a glass plate plus visual comparison
of unknown with standards gives an assessment of urinary
MMA excretion adequate for routine investigation.

In conclusion, the determination of MMA in
urine by the thin-layer chromatographic method developed
offered considerable improvement over the paper
chromatographic method. Among the advantages offered
by the thin-layer method are the following:

1. Shorter time required for development
because of the use of a one-dimensional system rather
than a two-dimensional one.

2. Better resolution of the components of
the extract.

3. Greater sensitivity. The maximum sensitivity
on the 250 micron silica gel plate using bromcresol green
is 10 μg. or less whereas on paper using the Altman reagent

it is 100 μg. or slightly less.

    4. Quantitative measurement of MMA levels in urine could be made.

4. <u>Procedure for the determination of urinary methylmalonic acid</u>

    The following is an outline of the method used for the determination of urinary MMA in the series of patients studied.

    1. Specimen: A twenty-four hour urine collection was made and an aliquot of the specimen kept frozen until used.

    2. Extraction: Approximately 1% of the twenty-four hour urine volume was ether extracted as described and the extract reduced in volume to 0.5 ml.

    3. Semiquantitative determination: Using a 250 micron layer of silica gel on a glass plate varying quantities of the extract were applied (up to 50 μl.) and a visual comparison with standards after spraying with bromcresol green gave a semiquantitative estimate of the quantity of MMA present. When 50 μl. of a comparable extract of a 1% volume of normal urine is applied, no MMA can be detected.

    4. Quantitative determination: Here the Chromagram was used as previously described and the MMA containing strip scanned on the Chromoscan. The volumes of unknown extract to be applied were determined from the results of the 250 micron thick silica gel plate and were

chosen such that they contained between 20 and 80 μg.
of MMA.

Twenty-one samples were run in duplicate
including duplicate extractions. The standard deviation
based on the duplicate samples run was $\pm$ 9 mg. per twenty-
four hours as calculated by the method of Youden (24),
with a mean of 218 mg. per twenty-four hours. This gave
a coefficient of variability of $\pm$ 4%.

B. DETERMINATION OF URINARY AIC

The method used for the measurement of urinary
AIC was a slight modification of that of McGeer et al (25)
and is as follows.

125 ml. of a twenty-four hour urine collection
is saturated with salt, acidified to about pH 2 and the
volume measured to record the increase due to salt
saturation. This urine is extracted with five 40 ml. volumes
of ethyl acetate. Two 50 ml. aliquots of the aqueous
phase are made alkaline to pH paper with a 1 : 4 mixture
of concentrated ammonium hydroxide and pyridine. To one
is added 50 μl. (25 μg.) of a 60 mg./100 ml. solution of
AIC to give a parallel analysis for recovery of AIC.

Both aliquots are extracted for three minutes
with nine 26 ml. volumes of a 100 : 5 n-butanol - pyridine
mixture and the organic extracts for each combined. The
organic phases are acidified by the addition of 10 ml.
of concentrated sulfuric acid and shaken for three minutes.

The aqueous layers containing the AIC are removed and the butanol layers washed once with 10 ml. of 0.2N H_2SO_4 then six times with 5 ml. of 0.2N H_2SO_4. The washings are added to the aqueous phase to give a final volume of about 40 ml.

Two 2 ml. aliquots of each extract are analyzed colorimetrically for non-acetylable diazotizable amines by coupling with N(1-naphthyl)-ethylene diamine. To the first aliquot in a tube of 22 mm. diameter is added 0.1 ml. of acetic anhydride and to the second 0.3 ml. of acetic anhydride. After vigorous shaking the tubes are allowed to stand for twenty minutes. 3 ml. of 0.2N H_2SO_4 is added to the first tube and 2.8 ml. to the second and then 0.5 ml. of 0.1% sodium nitrite is added to both. Exactly two minutes later, 0.5 ml. of 0.5% ammonium sulfamate is added followed immediately by 0.5 ml. of 0.1% N(1-naphthyl)-ethylene diamine. The mauve color which develops is read at 540 mμ. at ten minute intervals until a peak reading is obtained (usually within one hour). An extract blank is also run which is exactly comparable except that the N(1-naphthyl)-ethylene diamine is omitted and 0.5 ml. distilled water added instead.

Standards containing 3, 6, 9 and 12 μg. AIC (0.5, 1.0, 1.5 and 2.0 ml. of a 0.6 mg./100 ml. solution of AIC in 0.2N H_2SO_4 plus 0.2N H_2SO_4 to give a total volume of 2 ml.) are similarly analyzed and read at ten minutes against a reagent blank.

Quantities of AIC in the extracts are obtained by comparison with the standards. Appropriate calculations give the twenty-four hour urinary excretion of AIC. Normal values as quoted by McGeer are 0.98 ± 1.16 (2 S.D.) mg./day with a range of 0.3 to 2.5 mg./day.

IV. CLINICAL STUDIES

A. METHYLMALONIC ACID

1. Introduction

Urinary MMA excretion was determined on a total of 91 patients with a variety of clinical conditions. Many had findings which pointed to a possible deficiency of vitamin B_{12}. The results obtained are tabulated in Table I with detailed information on patients with laboratory or clinical evidences indicative of vitamin B_{12} deficiency. In addition other pertinent laboratory findings, where available, are included.

In the early patient studies, the MMA excretion was determined only by paper chromatography. These results are recorded as "positive" (MMA present) or "negative" (no MMA detected). Subsequently, thin-layer chromatography was used in addition to, or instead of, paper chromatography. These results are recorded as "less than" a certain number of milligrams per twenty-four hours rather than negative or as a quantitative result. It was found that so called "negative" results (the normal situation) represented values of less than 10 mg. MMA per twenty-four hours.

Serum vitamin B_{12} levels were determined by microbiological assay with the use of Euglena gracilis, as described by Ross (26). Normal levels are from 140 to 520 $\mu\mu$g. per ml. Serum folic acid levels were measured by the method of Herbert (27) using Lactobacillus casei. Normal serum folic acid levels are in excess of 3 mμg. per ml. Qualitative urinary formiminoglutamic acid (FIGLU) after a loading dose of 15 g. L-Histidine was determined by the method of Kohn et al (28). The measurement of urinary FIGLU after histidine loading recently has been studied as an indicator of folic acid deficiency. The conversion of FIGLU, a metabolite of histidine, to glutamic acid involves the transfer of the formimino group to tetrahydrofolic acid. Thus, in a deficient state, the transfer reaction is diminished with the subsequent excretion of increased amounts of FIGLU ("positive" result). In the normal condition, no FIGLU is detected ("negative" result). Co^{60}-labelled vitamin B_{12} absorption tests (Schilling tests) were performed using 0.5 μc. Co^{60}-vitamin B_{12}. Normal urinary excretion is in excess of 6% of the oral dose in twenty-four hours. The other laboratory tests, hemoglobin, hematological examination (peripheral blood and bone marrow smears) and gastric analyses were performed by standard procedures.

From one patient who was excreting increased quantities of MMA, three consecutive eight hour collections were taken in order to observe whether or not the MMA

TABLE I

Laboratory data of patients studied

Patient	Sex Age	Hemoglobin g./100 ml.	Hematological Findings	Free Gastric HCl	Urine FIGLU	Serum Folic Acid mμg./ml.	Serum Vit. B_{12} μμg./ml.	$Co^{60}B_{12}$ Absorption without IF %	with IF %	Urine MMA mg./24 hr.	Urine AIC mg./24 hr.	Neuro-logical symptoms	Diagnosis
#1	F48	6.8	macrocytic megaloblastic anemia	neg	---	---	---	1.2	---	146	0.9	slight	pernicious anemia
#2	F52	12.9	normal	neg	---	---	---	0.9	10	pos	---	severe	pernicious anemia
#3	M84	13.7	normal	neg	---	---	45	---	4.1	neg	---	severe	pernicious anemia
#4	F68	7.1	megaloblastic anemia	neg	pos	---	40	0.8	6.1	36	0.7	normal	pernicious anemia
#5	F56	5.9	macrocytic megaloblastic anemia	neg	---	---	35	0.2	3.1	34	1.1	---	pernicious anemia
#6	M74	8.0	macrocytic megaloblastic anemia	neg	pos	---	55	0.2	---	73	1.1	slight	pernicious anemia
#7	M84	2.3	macrocytic megaloblastic anemia	---	---	---	60	---	---	pos	---	slight	pernicious anemia

TABLE I (cont'd)

Patient	Sex Age	Hemo-globin g./100 ml.	Hematological Findings	Free Gastric HCl	Urine FIGLU	Serum Folic Acid mμg./ml.	Serum Vit. B12 μμg./ml.	Co60 B12 Absorption without IF %	with IF %	Urine MMA mg./24 hr.	Urine AIC mg./24 hr.	Neuro-logical symptoms	Diagnosis
# 8	M90	8.2	moderate macrocytic normoblastic anemia	neg	pos	---	---	0.3	---	53	1.1	normal	pernicious anemia
# 9	F51	14.3	normal	neg	---	---	---	0.8	5.7	1680	0.8	severe	pernicious anemia
# 10	M73	11.2	normal	neg	---	---	---	0.7	5.8	620	1.8	slight	pernicious anemia
# 11	F86	12.7	macrocytic anemia	---	---	---	40	2.5	14	77	0.5	normal	pernicious anemia
# 12	M75	---	macrocytic anemia	---	---	---	---	0.25	---	60	0.45	---	pernicious anemia
# 13	M71	6.4	megaloblastic erythropoiesis	neg	---	5.7	43	0.1	---	106	0.4	---	pernicious anemia
# 14	M68	9.5	macrocytic anemia - slight megaloblastic erythropoiesis	neg	---	7.0	82	1.5	---	150	1.4	normal	pernicious anemia

TABLE I (cont'd)

Patient	Sex Age	Hemo-globin g./ 100 ml.	Hematological Findings	Free Gastric HCl	Urine FIGLU	Serum Folic Acid mμg./ ml.	Serum Vit. B_{12} μμg./ ml.	$Co^{60}B_{12}$ Absorption without IF %	$Co^{60}B_{12}$ Absorption with IF %	Urine MMA mg./ 24 hr.	Urine AIC mg./ 24 hr.	Neuro-logical symptoms	Diagnosis
#15	M 3	5.9	macrocytic megaloblastic anemia	pos	pos	24	40	3.4	4.5	280	0.15	normal	pernicious anemia of infancy
#16	M64	9.1	macrocytic megaloblastic anemia	---	---	1.9	40	1.5	---	260	2.5	normal	pernicious anemia
#17	M82	6.4	macrocytic megaloblastic anemia	---	pos	7.6	<20	3.2	---	736	3.0	slight	pernicious anemia
#18	M84	7.9	moderate macrocytic megaloblastic anemia	neg	pos	5.3	<25	0.2	---	75	0.9	slight	pernicious anemia
#19	M42	14.1	slight macrocytosis & hypersegment-ation	neg	neg	4.7	65	0.5	5.0	45	1.8	severe	pernicious anemia
#20	F51	7.3	moderate pancytopenia	neg	neg	>40	<20	0.7	---	47 (5 hr.)	10.0 (5 hr.)	severe	pernicious anemia
#21	M--	---	megaloblastic anemia	---	pos	5.5	<60	0.7	---	12	1.2	---	pernicious anemia

TABLE I (cont'd)

Patient	Sex Age	Hemo-globin g./100 ml.	Hematological Findings	Free Gastric HCl	Urine FIGLU	Serum Folic Acid mμg./ml.	Serum Vit. B$_{12}$ μμg./ml.	Co^{60}B$_{12}$ Absorption with out IF %	with IF %	Urine MMA mg./24 hr.	Urine AIC mg./24 hr.	Neuro-logical symptoms	Diagnosis
#22	F60	5.5	macrocytic megaloblastic anemia	neg	pos	8.0	<20	2.6	6.0	12	1.4	normal	pernicious anemia
#23	F80	---	macrocytic megaloblastic anemia	---	---	---	20	2.9	---	186	0.85	---	pernicious anemia
#24	M77	11.6	macrocytic partially megaloblastic anemia	neg	---	---	<40	2.6	---	55	1.6	slight	pernicious anemia
#25	F68	14.4	normal	neg	---	---	---	0.35	1.5	neg	---	slight	diabetes mellitus cerebro-vascular disease vit. B$_{12}$ deficiency
#26	M52	15.6	normal	pos	---	---	---	2.5	1.7	neg	---	slight	cervical spondylosis ?? vit. B$_{12}$ deficiency

TABLE I (cont'd)

Patient	Sex Age	Hemo-globin g./100 ml.	Hematological Findings	Free Gastric HCl	Urine FIGLU	Serum Folic Acid mμg./ml.	Serum Vit. B12 μμg./ml.	Co60 B12 Absorption without IF %	Co60 B12 Absorption with IF %	Urine MMA mg./24 hr.	Urine AIC mg./24 hr.	Neuro-logical symptoms	Diagnosis
#27	F73	11.5	normal	---	---	---	---	2.4	---	<8	2.7	---	lobar pneumonia malabsorption
#28	M70	8.8	moderate hypochromic anemia	neg	pos	6.1	65	5.3	---	<6	1.1	normal	malabsorption syndrome with vit. B12 & iron deficiency
#29	M81	12.7	normal	---	---	2.6	45	3.4 (vol. low)	---	neg	---	---	carcinoma of stomach - inoperable
#30	M36	11.4	normochromic some poikilocytosis	---	pos	1.4	275	6.2	---	<12	6.5	normal	celiac sprue secondary osteomalacia iron & folic acid deficiency anemia

TABLE I (cont'd)

Patient	Sex Age	Hemo-globin g./100 ml.	Hematological Findings	Free Gastric HCl	Urine FIGLU	Serum Folic Acid mμg./ml.	Serum Vit. B$_{12}$ μμg./ml.	Co^{60}B$_{12}$ Absorption without IF %	Absorption with IF %	Urine MMA mg./24 hr.	Urine AIC mg./24 hr.	Neuro-logical symptoms	Diagnosis
#31	M9	11.8	slight to moderate hypochromia	---	pos	6.6	<18	3.1	---	<10	5.0	normal	idiopathic sprue diabetes mellitus
#32	M76	11.6	slight normoblastic hyperplasia	---	---	---	---	11.5	---	<10	5.2	normal	aplastic anemia diffuse interstitial fibrosis
59 patients										<10	normal in 29 patients		"miscellaneous"

TABLE II

Urinary MMA levels in three consecutive

eight hour collections from one patient

Patient	Time	MMA mg./24 hr.
#24	12 midnight - 8 am.	26
	8 am. - 4 pm.	13
	4 pm. - 12 midnight	17

TABLE III

Urinary MMA levels before and after

vitamin B_{12} therapy

Patient	Day	Intramuscular Dose of Vit. B_{12}	MMA mg./24 hrs.
# 9	1	--	1680
	4 months	after repeated doses of 1000 µg.	< 10
#16	1	--	260
	2	1000 µg.	
	3	400 µg.	
	5	400 µg.	
	7	400 µg.	
	12	400 µg.	
	13	--	< 10
#17	1	--	736
	3	1000 µg.	
	8	500 µg.	
	10	--	10
#24	1	--	54
	2	--	56
	5	1000 µg.	
	9	--	19
	12	1000 µg.	
	13	--	21
	14	1000 µg.	
	16	1000 µg.	
	19	--	< 5

excretion varied throughout the day. These results are shown in Table II.

On four patients MMA excretion was again measured after injections of vitamin B_{12} had been given. Specimens were collected from one of these patients at frequent intervals until MMA had disappeared from the urine. Table III gives these results.

2. Results

In Table I are the results obtained from a study of twenty-four patients on whom a diagnosis of pernicious anemia was made. Of this group, sixteen out of twenty-one patients tested had lower than normal hemoglobin levels and nineteen of the twenty-four showed macrocytosis or megalo-blastic erythropoiesis on the peripheral blood or bone marrow film. Serum vitamin B_{12} levels determined on eighteen of the patients were well below the lower limits of normal. Co^{60}-vitamin B_{12} absorption as done on twenty-two of the patients was also much reduced. Histamine-fast achlorhydria was a finding in sixteen of seventeen patients tested, the one patient showing free gastric acid being a three year old with a diagnosis of "pernicious anemia of infancy".

Urinary methylmalonic acid excretion on all but one of the patients was found to be elevated. This patient, #3, had been under treatment for pernicious anemia until two years prior to being investigated early in this study. His serum vitamin B_{12} level was low, 45 $\mu\mu$g. per ml., his vitamin B_{12} absorption even with intrinsic factor was

slightly low and his urinary MMA excretion was "negative".
In twenty-one of the remaining twenty-three patients of
this group on whom urinary MMA was quantitated, the twenty-
four hour excretion ranged from 10 to 1,680 mg., the normal
excretion being less than 10 mg. per day.

Patients #25 to #29 had one or more laboratory
findings which suggested vitamin B_{12} deficiency. Patients
#25 and #26 had a decreased vitamin B_{12} absorption which
did not increase when repeated with intrinsic factor.
Patient #25 had a secondary diagnosis of vitamin B_{12}
deficiency while for patient #26 the same diagnosis was
queried. The urinary MMA excretion in both these patients
was "negative" as determined early in the series by
qualitative paper chromatography prior to the development
of the thin-layer quantitative method. Both patients had
a normal hematological picture.

Patients #27 and #28 had a diagnosis of mal-
absorption. Patient #27 had a low vitamin B_{12} absorption.
Patient #28 had a normal vitamin B_{12} absorption but a low
serum vitamin B_{12} level. In both cases MMA excretion was
normal. Patient #29, diagnosed as inoperable carcinoma of
the stomach, had a low serum vitamin B_{12} level but a
"negative" MMA excretion.

Patients #30, #31 and #32 are included in Table I
as they had elevated urinary MMA levels. These patients
will be discussed later.

The remaining fifty-nine cases represent hospital patients from medical and surgical wards with a wide variety of disorders other than vitamin B_{12} deficiency and make up the "miscellaneous" group in Table I. Included in this group were patients with iron and folic acid deficiency anemias, malabsorption showing no evidence of vitamin B_{12} deficiency, pneumonia and many other conditions. This group was assessed to study the specificity of urinary MMA excretion as an index of vitamin B_{12} deficiency and to observe any false positive results in disorders not related to a deficiency of vitamin B_{12}. No increase in MMA excretion above normal was observed in this group.

The neurological symptoms have been tabulated in Table I in three rather broad groups; normal, slight (including minor neurological involvement such as numbness and/or tingling in the extremities) and severe (being subacute combined degeneration of the spinal cord).

Table II shows the eight-hourly MMA excretion of the one patient studied to observe any variation within the day in urinary MMA. In this particular patient the overnight collection showed a greater amount of MMA excreted than either of the other two eight-hour periods neither of which differed appreciably from the other.

As is shown in Table III, MMA excretion returns to normal levels within a few days of the patient receiving intramuscular injections of vitamin B_{12}. Patient #9 was not followed closely but after a four month period during

which she received a series of intramuscular injections of
vitamin B_{12}, the twenty-four hour MMA excretion dropped
from 1,680 mg. to less than 10 mg. In the other three
cases, the MMA excretion returned to normal within ten
days to two weeks.

3. Discussion

All but one of the patients on whom a diagnosis
of pernicious anemia was established showed an elevation
of urinary MMA. As has been stated, the one patient on
whom MMA was recorded as "negative" was assessed using
the paper chromatographic procedure and may possibly have
shown a slightly increased level of MMA which would not
have been detected under the early and less sensitive
conditions of this procedure. In all other cases of
pernicious anemia, MMA excretion served equally as well
as the Co^{60}-vitamin B_{12} absorption as a measure of vitamin
B_{12} deficiency. Like labelled B_{12} absorption, urinary MMA
may be abnormal before there is hematological evidence of
the vitamin deficiency. This is evidenced by those
patients in the series who showed no anemia or megalo-
blastic erythropoiesis yet had decreased vitamin B_{12}
absorption and increased MMA excretion.

In considering the urinary MMA levels and the
neurological symptoms shown by the patients with pernicious
anemia, there would appear to be no correlation between the
two. There is no method for determining blood MMA, but
assuming MMA excretion to reflect body levels of MMA, an

increased quantity of circulating MMA apparently does
not relate to the degree of neurological involvement. One
patient with subacute combined degeneration of the spinal
cord had an MMA level of 1,680 mg. per twenty-four hours
while another with the same condition had an MMA level of
45 mg. per twenty-four hours. On the other hand, patients
with no neurological disorders had MMA levels ranging from
12 to 280 mg. per twenty-four hours. This is consistent
with previous findings that other measures of vitamin B_{12}
deficiency do not correlate with the degree of neurological
involvement.

A sodium propionate loading test was considered
on the basis that biochemically speaking, this could be a
useful procedure for revealing mild deficiencies of body
levels of vitamin B_{12} that might not appear from clinical
and other observations. However, the reports of White
and Cox (2) and of Kahn and coworkers (29) in the very
recent literature (July, 1965) of attempts using this
approach were not encouraging so this was not carried out.

While no conclusions can be drawn from the
results on only one patient, the variation in MMA excretion
in consecutive eight hour specimens in this patient is in
agreement with the observations of Kahn et al (29). This
group found there could be considerable variation in the
amount of MMA excreted from one eight hour period to the
next.

The observed drop in MMA excretion after administration of vitamin B_{12} to the patient is an expected finding and demonstrates that it is biologically valid to relate MMA excretion and B_{12} levels and that increases are not merely spurious findings.

B.   4(5)-AMINO-5(4)-IMIDAZOLECARBOXAMIDE

1.   Introduction

Urinary AIC was determined on forty-nine patients, twenty of whom had a diagnosis of pernicious anemia. The results on those patients with pernicious anemia are included in Table I along with those on four other patients who showed an increased level of urinary AIC. AIC was also determined on three of the patients on whom MMA estimations were done after treatment with vitamin B_{12}. These results are in Table IV.

2.   Results

Of the group of twenty patients with pernicious anemia, only three had elevated levels of urinary AIC. Among the remaining twenty-nine cases, four had higher than normal levels of AIC. Three of these four patients suffered from malabsorption while the fourth had an aplastic anemia.

The three patients recorded in Table IV all showed an appreciable drop in their urinary AIC levels after vitamin B_{12} therapy.

TABLE IV

AIC excretion before and after treatment with

intramuscular injections of vitamin B_{12}

Patient		AIC mg./24 hrs. (Normal < 2.1 mg./24 hrs.)
#16	Before	2.5
	After	1.0
#17	Before	3.0
	After	0.8
#24	Before	1.6
	After	0.9

3. Discussion

The results of this study on twenty patients do not show increased urinary AIC excretion to be a constant feature of pernicious anemia and reduced B_{12} levels as evidenced from depressed isomerization of methylmalonyl-CoA to succinyl-CoA. If indeed vitamin B_{12} is directly involved in the folic acid dependent formylation of AICAR to FAICAR rather than merely being necessary for the primary activity of folic acid, this enzymatic conversion is still operative at low levels of B_{12} availability and in the presence of clinical evidence of B_{12} deficiency. Thus AIC excretion does not appear to be a reliable measure of B_{12} deficiency. However, it should be noted that other authors studying smaller series of patients with B_{12} deficiency have shown an increased AIC excretion. Luhby and Cooperman (4) on finding urinary AIC to be elevated in vitamin B_{12} deficiency states but not in folic acid deficiencies interpreted this as indicating that deficiency of vitamin B_{12} alone specifically results in increased AIC excretion. Herbert (5) and Middleton (6) found increased urinary AIC in both B_{12} and folate deficiencies and these authors suggest that folic acid deficiency alone produces the abnormally high urinary excretion of AIC. They conclude that as vitamin B_{12} is thought to be necessary for the normal activity of folic acid, the similar AIC findings in vitamin B_{12} deficiency alone are not unexpected. It is interesting that in those

patients on whom AIC was measured before and after they received vitamin B_{12} there is an appreciable drop in AIC excretion after treatment. Thus on this basis it would appear that there is some validity to the proposal that B_{12} is involved either primarily or secondarily in the formylation of AICAR to FAICAR.

V. CONCLUSION

1. A quantitative method for the determination of MMA in urine has been developed which uses the technique of thin-layer chromatography following an ether extraction of urine. By this method normal urinary excretion of MMA is less than 10 mg. per day and the coefficient of variability is four percent.

2. In twenty-three cases of clinically proven pernicious anemia, the urinary MMA excretion was increased in all cases. Thus, measurement of MMA excretion is a sensitive index of vitamin B_{12} deficiency. Urinary MMA returns to normal in response to vitamin B_{12} therapy.

3. All cases with a proven diagnosis of pernicious anemia and low Co^{60}-vitamin B_{12} absorption (Schilling test) also exhibited increased urinary MMA levels. Thus for laboratories not licensed for use of radioisotopes necessary for the Schilling test, urinary MMA measurement should be considered to be a reliable and adequate substitute in the laboratory diagnosis of pernicious anemia.

4. The degree of neurological involvement in pernicious anemia appears to be independent of the elevated

body levels of MMA as evidenced by MMA excretion.

5. The excretion of MMA in patients with pernicious anemia may be elevated before anemia or the abnormal hematological features develop. This aspect should be an aid to the early diagnosis of pernicious anemia.

6. Urinary excretion of MMA in successive eight hour periods as measured in one patient was variable. This finding concurs with reports of others who have studied the diurnal excretion of MMA.

7. Urinary AIC was only occasionally increased in those patients with pernicious anemia and the study does not give any further information on the possible direct involvement of vitamin B_{12} in the reaction AICAR to FAICAR, a reaction involved in the synthesis of the nucleotide, inosinic acid. Although it is well established that vitamin B_{12} deficiency leads to disordered nucleic acid synthesis, results in this study do not show clearly the involvement of vitamin B_{12} in the AICAR to FAICAR step.

VI. BIBLIOGRAPHY

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